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HISTOCHEMISTRY OF CYTOPLASMIC RESERVES IN EXCIPULAR HYPHAE OF *CATILLARIA BOUTEILLEI* (DESM.) ZAHLBR.

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SUMMARY. – Histochemical aspects of cytoplasmic reserves in excipular hyphae during the development of apothecia in *Catillaria bouteillei* were studied by conventional, fluorescence and scanning electron microscopy. Positive tests were obtained for polysaccharides, proteins, polyphosphates, lipids and acid phosphatase activity. Our results show that the excipular border can be distinguished in two separate parts of differing histochemical reactivity. While the lateral and basal portions are an important site for the accumulation of reserve substances, active meroholocrine secretion takes place in the upper portion. The extrusion process allows the appearance in the adult apothecia of a thin, intensely acidic layer of mucilage, above the hymenium.

RÉSUMÉ. – Les aspects histochimiques des réserves cytoplasmiques des hyphes excipulaires pendant le développement des apothécies chez *Catillaria bouteillei* sont étudiés par microscopie photonique traditionnelle, à fluorescence et électronique à balayage. Les tests sont positifs pour les polysaccharides, les protéines, les polyphosphates, les lipides et l'activité phosphatase acide. Nos résultats montrent que la marge excipulaire est constituée de deux parties ayant des réactions histochimiques différentes. Tandis que les parties latérales et basales constituent un site important d'accumulation de substances de réserve, la partie supérieure présente une sécrétion de méroholocrine active. Le processus d'extrusion dans l'apothécie adulte conduit à l'apparition d'une fine couche de mucilage très acide au-dessus de l'hyménium.

KEY WORDS : *Catillaria bouteillei*, lichenized fungi, histochemistry.

INTRODUCTION

Catillaria bouteillei (Desm.) Zahlbr., a foliicolous lichen, was the subject of our recent study concerning some histochemical aspects of the hypothalline hyphae (MODENESI & al., 1986). Our results have allowed the correlation of the presence of mucilage on the lower edge of the thallus with the particular ecology of the lichen. During this study it was observed that the remarkable

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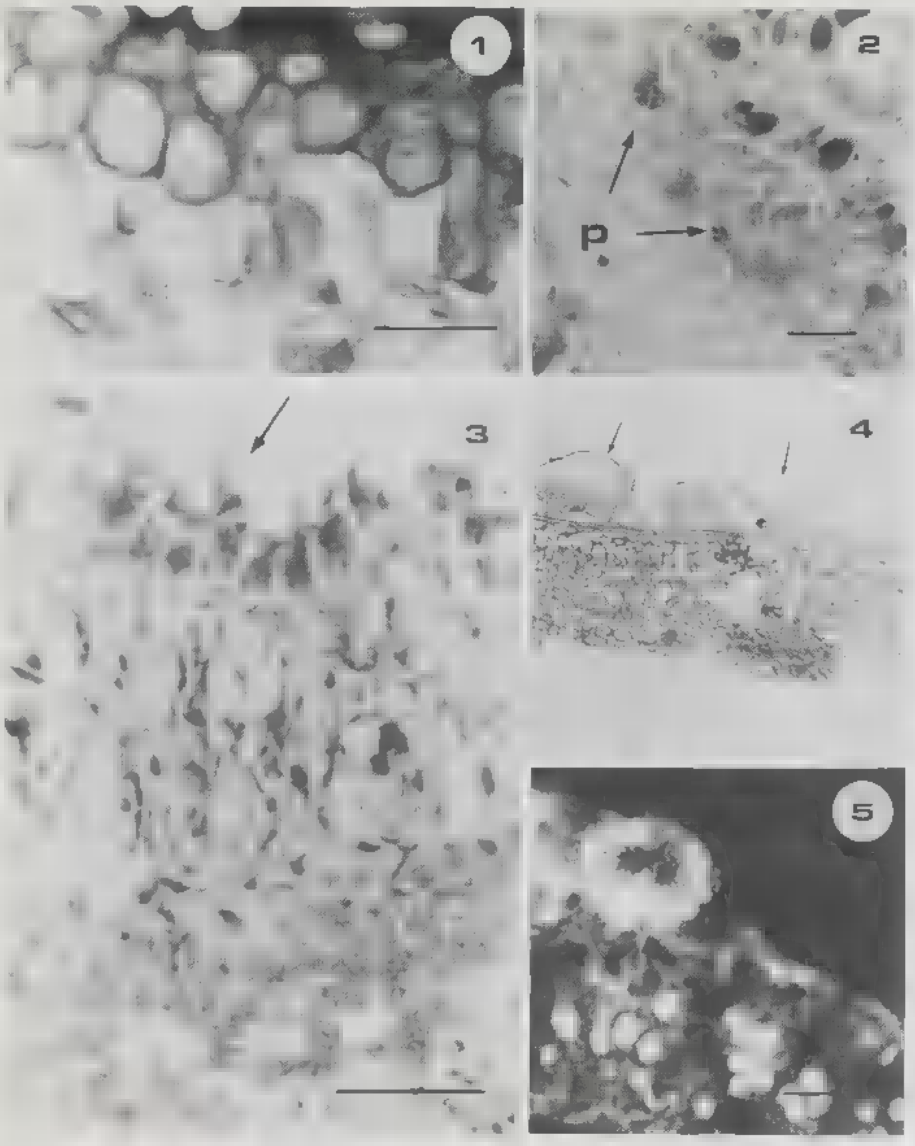


Figures 1 to 5 — 1 : Section of basal excipular hyphae, of an apothecium in phase 1, showing extensive deposits of polysaccharides and punctuated unstained areas. PAS reagents. Scale bar 10 μm. 2 : Section of basal excipular hyphae of an apothecium in phase 2 stained with TBO pH 4.4. Polyphosphates granules (p) of varying size are visible as metachromatic (arrows). Scale bar 10 μm. 3 : Section of apothecium (central part) in phase 1 stained with Amido black. Round protein-containing structures in the basal portion of excipulum are common. In the upper portion the staining reaction is most extensive. Note the lysis of some upper excipular cells (arrow). Scale bar 20 μm. 4 : Transversal section of the lichen-carrying leaf showing in the upper edge of sectioned apothecia (phase 2) a thin metachromatic layer (arrows) of sulphated polysaccharides. TBO pH 1. Scale bar 100 μm. 5 : Transversal section of the lichen thallus carrying an apothecium in phase 1. The excipulum intensely fluoresces silvery-white with phosphine. Scale bar 10 μm.

histochemical reactivity of the cytoplasm of the excipular hyphae, the tissue surrounding the thecium and evident as the proper margin, varied during the transition from the youthful phase to the adult phase in the apothecia.

To understand better the changes which may occur at a cellular level, a histochemical study of apothecia as they developed must be undertaken. In this respect such methods have provided a valuable tool in the study of some fungi (MOTTA, 1969; KOSAHIH & WILLETS, 1975; MOORE-LANDECKER, 1981). On the other hand histochemical observations in the lichenized-fungi are not extensive. Our study carried out by conventional, fluorescence and electron scanning microscopy, concerns histochemical aspects of excipular hyphae contents during the final development of apothecia in *Catillaria bouteillei*.

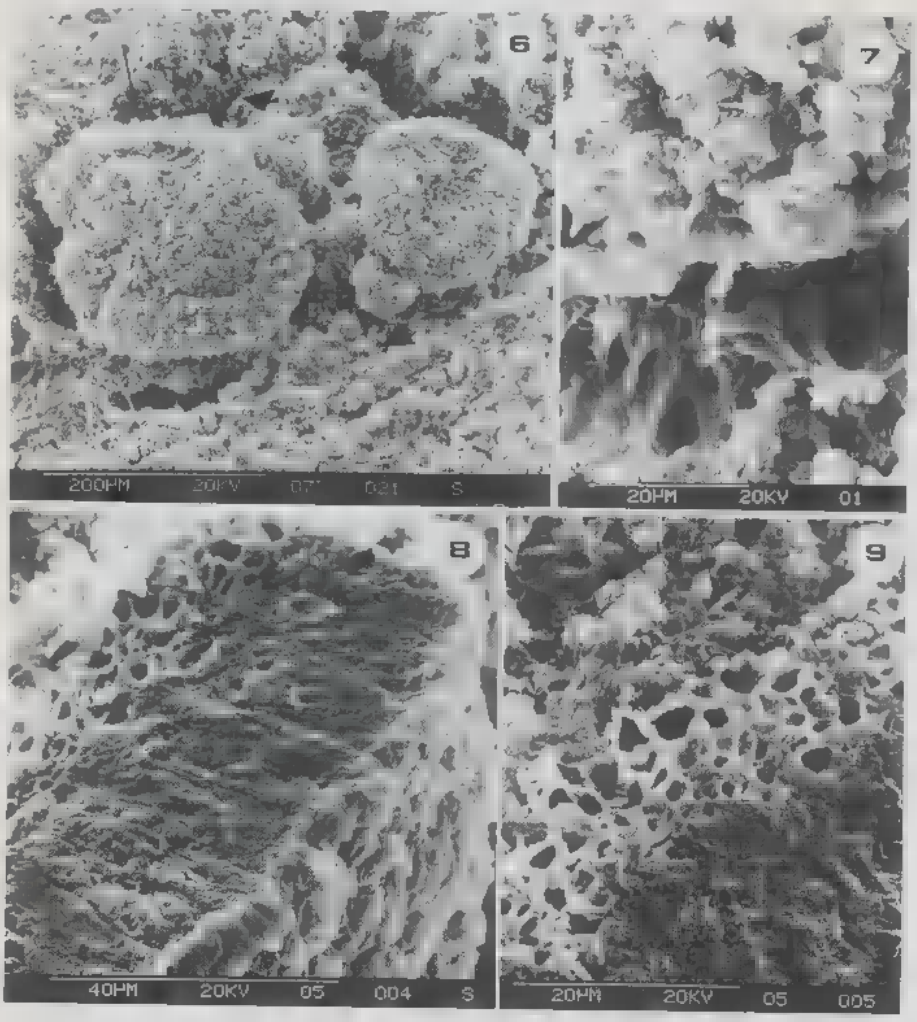
MATERIAL AND METHODS

Thalli of *Catillaria bouteillei* growing on leaves of *Buxus sempervirens* were collected in eastern Liguria.

Light microscopy : small pieces of leaf-carrying lichen or isolated apothecia were fixed either in FAA (Formalin-Acetic acid-ethyl Alcohol) (SASS, 1958) for 24 hours or in glutaraldehyde 3 % in 0.1 M phosphate buffer at pH 6.8 for 4 hours at 0-4°C. Following dehydration in an ethanol series, samples were embedded either in JB4 resin (Polyscience Inc.) or in methyl-butyl methacrylate mixture (FEDER & O'BRIEN, 1968) in embedding polyethylene (Polyscience Inc.) capsules. For the histochemical demonstration of acid phosphatase, specimens were fixed and embedded in JB4 at cold temperature according to NAMBA & al. (1983).

Sections 2.5 µm thick were cut with a glass knife on a Reichert OM2 microtome. For histochemical investigations, embedded sections or sections obtained by a cryostat microtome were processed with the following methods : a) Periodic acid-Schiff reagent (PAS) for polysaccharides (PEARSE, 1985); b) Carmine Best's method (PEARSE, 1985) and Iodine-Potassium iodide reaction (IKI) (JENSEN, 1962) for glycogen; c) Toluidine Blue 0 (TBO) at pH 4.4 and pH 1

Figures 1 à 5 – 1 : Coupe d'hyphes excipulaires basales d'une apothécie en phase 1, montrant des dépôts importants de polysaccharides et des zones ponctuelles non colorées. Réaction PAS. Échelle : 10 µm. 2 : Coupe d'hyphes excipulaires basales d'une apothécie en phase 2, colorée avec le TBO pH 4,4. Les granules de polyphosphates (p) de tailles variables sont métachromatiques (flèches). Échelle : 10 µm. 3 : Coupe d'apothécie (partie centrale) en phase 1, colorée à l'amido black. Les structures rondes contenant des protéines sont fréquentes dans la partie basale de l'excipulum. Dans la partie supérieure, la réaction de coloration est plus forte. Noter la lyse de quelques cellules excipulaires supérieures (flèche). Échelle : 20 µm. 4 : Coupe transversale de la feuille portant le lichen, montrant sur le bord supérieur de l'apothécie sectionnée (phase 2) une couche fine métachromatique (flèches) de polysaccharides sulfatés. TBO pH 1. Échelle : 100 µm. 5 : Coupe transversale du thalle lichénique portant une apothécie en phase 1. L'excipulum montre une intense fluorescence blanc-argenté avec la phosphine. Échelle : 10 µm.



Figures 6 to 9 – SEM micrographs. 6 : Upper view of the thallus with apothecia at various stages of development. In the adult one (arrow) the excipulum is not found on the upper side; in the youngest ones it occurs = a complete border (head arrow). 7 : Lateral view of an adult apothecium showing the excipular edge. Here the cells have a spongy aspect due to the lytic processes. 8 : Transversal view of an adult apothecium. The excipulum only basally occurs. 9 : Transversal view of the basal excipular cells.

Figures 6 à 9 – Microscopie électronique à balayage. 6 : Vue «aérienne» du thalle avec des apothécies à différents stades de développement. Sur les apothécies adultes (flèche), l'excipulum ne se trouve pas sur la partie supérieure; sur les plus jeunes, il forme une marge complète (tête de flèche). 7 : Vue générale d'une apothécie adulte montrant la marge excipulaire. Les cellules ont ici un aspect spongieux, dû aux procédés lytiques. 8 : Vue transversale d'une apothécie adulte. L'excipulum apparaît seulement à la base. 9 : Vue transversale des cellules excipulaires basales.

for polyanions (LING LEE & al., 1977); d) Alcian Blue 8GX (AB) at pH 0.5 for acid sulphated polysaccharides (LEV & SPICER, 1964); e) Histochemical experiments with critical electrolyte concentration (CEC) for blocking alcianophilia at graded concentrations of $MgCl_2$ to show the various charged groups of carbohydrates (PEARSE, 1985); f) Amino Black 10 B for proteins (FISHER, 1968); g) Sudan Black B and Phosphine 3R for lipids (PEARSE, 1985); h) BURSTONE's method for acid phosphatase (NAMBA & al., 1983). The following extraction and enzymatic digestion procedures were carried out on sections : a) Trichloroacetic acid (TCA) prior to staining with TBO at pH 1 to remove polyphosphate (ASHFORD & al., 1975); b) Papain, prior to staining with Amido Black to remove protein (PEARSE, 1985); c) α-amylase from *Bacillus subtilis* or human saliva (BULLOCK & al., 1980) prior to staining with PAS reagent to remove glycogen.

For all the above histochemical methods, control reactions were carried out following the suggestions of the respective authors. For fluorescence microscopy, after staining, sections were mounted in an UV-inert medium (Serva Inc.) and observed with a Leitz microscope fitted with an epi-illuminator, UV exciting illumination and Fluotar objectives.

Scanning electron microscopy : samples were prepared as described elsewhere (MODENESI & al., 1986). To obtain transverse section views of apothecia, isolated apothecia were frozen by immersion in liquid nitrogen and fractured with a razor blade. Specimens were then air dried, directly coated in the Sputtering and observed with a Stereoscan Cambridge 250 MK2.

RESULTS

Lecideoids apothecia of *Catillaria bouteillei* show a whitish paraplectenchymatous border called excipulum (SANTESSON, 1952) of 10-30 µm in thickness. It is made up of sub-roundish cells in section of 4-8 µm in width (Figs. 1, 9).

During the phases of development of the apothecia observed, the excipulum shows itself in two different configurations : a) it forms a complete border around the fertile part, covering it on all sides (phase 1 or youthful phase) (Figs. 3, 5, 6); b) it is absent on the upper side because of the growth of the underlying fertile portion, and in part because of the lysis of the upper excipular cells (Fig. 3, 7). In this way the excipulum only remains in the lateral and basal portions (phase 2 or adult) (Figs. 4, 6, 8).

Our histochemical observations, summarized in Table 1, refer to the content of excipular hyphae in these two different stages of the development of the apothecia.

Carbohydrates : the PAS reaction, which indicates the presence of polysaccharides with 1:2 glycol groups, stained red the cytoplasm of the excipular cells. This took on a characteristic punctuated aspect, due to the presence of minute and numerous unstained areas of a non-polysaccharide nature (Fig. 1). The Best

Stain	Substances	Observed reaction					
		Phase 1			Phase 2		
		B	L	U	B	L	Us
PAS	Polysaccharides	++	++	++	+	+	+
Best's/IKI	Glycogen	+	-	-	-	-	0
TBO pH 4.4	Polyanions	++	++	++	+	-	++
TCA-TBO pH 1	Polysulphates	0	0	++	0	0	++
AB pH 0.5	Sulphated-polysaccharides	0	0	++	0	0	++
Amido Black	Protein	++	++	+++	+	-	0
Phosphine	Lipids	+++	+++	+++	+	+	0
Sudan Black	Lipids	+++	+++	+++	+	+	0
Burstone	Acid phosphatase	+++	+++	+++	++	+	0

Legend: 0= no appreciable staining, += light staining, ++= moderate staining, +++= heavy staining, -= ambiguous staining. B, L, U= basal, lateral and upper part of excipulum. Us= amorphous layer secreted in upper part of excipulum.

Table 1 – Staining properties of the cytoplasm of the excipular cells in developing apothecia of *Catillaria bouteillei*.

Tableau 1 – Propriétés de coloration du cytoplasme des cellules excipulaires dans les deux phases du développement des apothécies de *Catillaria bouteillei*.

and IKI reactions gave slighter results, only weakly staining the cytoplasm, often with ambiguous results. Analogously the α-amylase or human saliva digestions only managed to remove a small part of the positive PAS matter. These results indicate the presence of a small quantity of glycogen in the cytoplasm which is occupied in greater quantity by other insoluble polysaccharides.

In young apothecia (phase 1) the intensity of the PAS reaction was equally moderate in all parts of the excipulum; in the adult apothecia (phase 2) the reactivity was maintained only in the basal part and in a thin amorphous line above the hymenium.

Polyanions : in phase 1 the cytoplasm showed minute metachromatic granules (bluish red) with TBO at pH 4.4 (Fig. 2). In these conditions several different polyanions (polyphosphates, polysulphates and polycarboxilic acids) carry a negative charge and would give a metachromatic reaction (BULLOCK & al., 1980).

Metachromasy persisted with TBO at pH 1, when the polysulphates and polyphosphates only still proved to be ionized (LING LEE & al., 1977). Staining with TBO at pH 1, preceded by digestion with TCA, which removes polyphos-

phates (ASHFORD & al., 1975), showed that the granules in the cytoplasm of the hyphae of the lateral and basal portions of the excipulum had been completely removed. However this removal did not take place in the upper excipular edge during phase 1. In this latter area the same results was obtained with AB at pH 0.5 which stain the mucopolysaccharide sulphates (Fig. 4). The presence of sulphate acid groups is further substantiated by the persistent alcianophilia with CEC procedure in the presence of 0.5-0.6 M $MgCl_2$.

In phase 2, TBO at pH 4.4 and pH 1, shows the presence of few polyphosphate metachromatic granules only in the basal portion of the excipulum. Both in this case as in the PAS reaction, the upper part of the hymenium shows a thin anamorphous metachromatic line with TBO at low pH, resistant to TCA and positive to AB at pH 0.5.

Protein : at phase 1 the excipular cells show a strong affinity with Amido Black, intensely staining a large number of round structures (Fig. 3). They were about the same size as the unstained areas in PAS stained sections of excipulum (Fig. 1). The removal of such structures brought about by protease preparation confirms that they contained proteins.

The protein storage bodies are clearly delimited in the cytoplasm, only in the basal and lateral portions of the excipular cells. In contrast, in the upper portion they are hardly visible, since also a large part of the cytoplasm reacts intensely with Amino Black and it is sensitive to the protease (Fig. 3). On reaching maturity (phase 2) the basal portion maintains its activity, while it decreases in the lateral portions and disappears in the upper part. This last fact shows that proteins and protein-complexes in histochemically detectable amounts are absent in the thin amorphous line above the hymenium.

Lipids : all parts of excipulum in young apothecia are intensely silvery white fluorescent under ultraviolet light with Phosphine (Fig. 5). Sudan Black also gave a positive reaction confirming a widespread presence of lipidic matter in the cytoplasm of the excipular hyphae.

In phase 2 the tests used show a general and remarkable decrease in affinity to the cytoplasmic content.

Acid phosphatase : tests to demonstrate this enzymatic activity have indicated that during the maturation process in apothecia, the excipular cells were particularly active. At phase 1 the cytoplasm is rapidly and intensely marked. At phase 2 only the basal cells maintain this activity.

DISCUSSION

Our results show that the excipulum in *Catillaria bouteillei* can be distinguished into two separate differentiable parts because of the histochemical reactivity of the cytoplasm constituent cells.

The basal and lateral portions are an important site for storage of reserves. These consist in polysaccharides, among which a small quantity of glycogen, and also proteins, polyphosphates and lipids. In these portions the total quantity of carbohydrates greatly decreases when the apothecia reach maturity. This fact is in keeping with the presence of a considerable acid phosphatase activity. FIGIER (1972) correlates this activity to phenomena of dephosphorilation which suggest an important active intercellular movement of glucids.

The other major cytoplasmic storage reserves are proteins in the form of protein bodies. This is a common feature in fungi where a high protein or protein body content goes together with the phases of the apothecia's development in ectal and medullary excipulum in *Pyronema omphalodes* (MOORE-LANDECKER, 1981) and in the developing sclerotia of *Sclerotinia minor* (BULLOCK & al., 1980). As these authors have noted, this occurrence is not surprising in sclerotia since a large nitrogen requirement would be expected in the initial stage of germination.

Our staining reactions suggest that phosphates are present in the form of polyphosphate. Polyphosphate granules have been previously reported from the fungal component in *Collema leucocarpon* and *Peltigera dolichorrhiza* (CHILVERS & al., 1978). According to HAROLD (1966), usually lower organism store this anion as polyphosphate. This is a thermodynamically high energy compound and its accumulation could be an energy storage mechanism (BULLOCK & al., 1980).

Other cytoplasmic components are lipids which in developing apothecia of *Catillaria bouteillei* are quickly mobilized product. Lipids have been reported as the most common storage product in fungi (HAWKER, 1965; CHET & al., 1977) and they are also reported in oil hyphae in an endolithic *Catillaria* sp. (KUSHNIR & al., 1978).

The upper excipular portion differs histochemically from the preceding ones because of the characteristic presence of sulphated mucopolysaccharides together with the other cytoplasmic reserves (Table 1). This portion is visible as cellularized, only in young apothecia; on reaching maturity they are removed laterally. Furthermore some of the cells are destroyed during growth.

Therefore it seems reasonable to assume that the thin amorphous line visible in adult apothecia is the result of the metabolic activity of the upper excipular cells. In fact it conserves positive reactivity for AB at pH 0.5 as well as the cytoplasmic content of those cells in phase 1 (Table 1). Thus this layer would appear to be derived both from the secreting merochrine processes (where the cells remain intact) and from the holochrine processes (which bring about the lysis of the secreting cells). A high acid phosphatase activity is visible in phase 1. This according to the various authors who consider it to be characteristic of the glandular cells secreting hydrophilic substances in higher (SCHNEPF, 1974; FIGIER, 1972) and lower (HÉBANT & BONNOT, 1974) plants.

In mature apothecia of *Catillaria bouteillei*, this intensely acid, mucilaginous layer is naturally capable of linking water in amounts directly related to the

intensity of the available negative charges (MODENESI & VANZO, 1986). This may therefore have the function of contributing to hymenium hydration, delaying drying and preventing excessive water loss in this foliicolous lichen which lives in strict aerohygrophilic conditions (SANTESSON, 1952; MARGOT, 1977). Another possible function of the mucopolysaccharide layer is a feature related to the covering of the outer surface with mucilage, acting as a first line of defence against colonization by pathogens (MODENESI & al., 1986; MODENESI & VANZO, 1986).

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